Investigating the role of bone marrow derived cells in the healing of chronic wounds.

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CERTIFICATE

This is to certify that ‘INVESTIGATING THE ROLE OF BONE MARROW DERIVED STEM CELLS IN THE HEALING OF CHRONIC WOUNDS’, submitted as a thesis for M.S. Degree Branch I –General Surgery examination of the Dr. M.G.R. Medical University of Tamil Nadu, is the bonafide work of the candidate –
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CERTIFICATE

This is to certify that the topic entitled 'INVESTIGATING THE ROLE OF BONE MARROW DERIVED STEM CELLS IN THE HEALING OF CHRONIC WOUNDS' is the bonafide work done by Dr. Prakash Jain, post graduate trainee in General Surgery at Christian Medical College, Vellore.

This work has been carried under my guidance and supervision in partial fulfilment of the regulation of Dr. M.G.R. Medical University of Tamil Nadu for Master of Surgery- Branch I (General Surgery) examination to be held in March 2009.

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Introduction

Wounds are a ubiquitous part of any surgical practice and are often challenging to treat. They can be broadly thought of as acute or chronic and can have varied etiologies (1, 2). Wounds, in one form or another, contribute to a significant burden on health care in terms of morbidity, cost of treatment and duration of healing (3,28).

In resource-rich nations, wound management has progressed significantly and some countries have shown that community based wound management teams can help to decrease the burden on hospitals (3). However, there has been no real progress in reducing the time required for wound healing (4).

Stem cells have the potential to reduce time to healing since they are able to differentiate into the various cell types required in wound healing (5, 6, 7, 8, 9). It is known that bone marrow derived stem cells have the potential to be recruited locally and to transform into keratinocytes and other cells promoting local granulation without cell-cell fusion (13, 14). It is therefore possible that research into this property of bone marrow derived stem cells could serve as an answer to the problem that has plagued surgeons for centuries: *How do we speed up wound healing?*

Disease burden:

It is estimated that the prevalence of wounds in the community is 15.0 per 1000 and chronic wound prevalence is 4.48 per 1000 (29). Diabetics in India in the over 20 age group are predicted to increase by 151% from the years 2000 to 2030 (30). Diabetics have 30 to 40 times greater chances of major complications compared to the normal population and 15% of diabetics are estimated to have foot ulcers at any given time (28). It is also estimated that 40 to 77 per 10000 diabetics have a major amputation in their life-time and that 65% of these could be avoided with adequate foot care (28). India will have the world's largest diabetic population by 2030 (30) and this will add a further load on our already burdened health system.

What makes an ulcer persist long enough to be considered non-healing or chronic and further, what can be done to improve the wound healing rates in such situations?

Chronic wounds have long been held as a difficult area in any general surgical practice. They are difficult to treat and outcomes tend to be poor. This is related to the multiple factors that make a wound chronic and keep it from healing.

The definition of a chronic wound varies in terms of duration and character of the wound. The most precise definition is that of a wound that has not shown any progress towards healing for more than or equal to one month (2, 27). However, this definition does not take into account the size of the wound or the underlying etiology of the wound.

Chronic wounds or ulcers have been the source of intense debate and much research (1, 4). Many factors have been implicated in preventing wound healing and others have been identified as pro-healing. However, there has been no magic bullet or single agent that has been isolated that either prevents or promotes wound healing. This implies that wound healing is a complex and active process and is multi-factorial and what causes a wound to become chronic is still not clear (31).

Aims & Objectives

Objective:

To improve current practice in the healing of chronic wounds by assessing a novel treatment option in a scientific manner.

Aim : 1) To determine if the rate of wound healing in chronic wounds can be augmented by the local application of stem cells derived from autologous concentrated bone marrow aspirates.

2) To standardize the methodology for the local application of autologous bone marrow derived stem cells in chronic wounds.

Study Type:

Randomized controlled study in a tertiary care centre in South India, Christian Medical College, Vellore.

Normal wound healing:

Wound healing can be defined as the restoration of the normal anatomic integrity and function of injured tissue (27). Tissue can be injured in many ways, including direct trauma, incisions from surgical wounds and even ulceration and tissue loss due to necrosis secondary to infections of various types.

Epithelium and liver tissue are known to regenerate; this is often limited by scarring and fibrous tissue. While fibrosis and scarring are considered as a part of healing, much of normal function may be lost and in fact, the healed area invariably has less function and aesthetic value than previously (19).

The processes involved in the healing of a wound can be simply summed up as (19, 27):

- Coagulation
- Inflammation
- Matrix synthesis & deposition
- Angiogenesis

- Fibroplasia
- Epithelialization
- Contraction
- Remodeling

Each of these processes can be further discussed and their roles have been studied in detail.

Coagulation:

As soon as blood vessels are damaged in the event that caused the injury, the coagulation pathway is initiated. In this manner the wound healing activities begin almost simultaneously with the wounding event. Local activation of phospholipase A2 at the cell membrane causes the metabolism of arachidonic acid to the eicosanoid precursors and then to the prostaglandins and the leukotrienes. Further, the hemostatic events such as vascular constriction, platelet plug formation and fibrin clot deposition also occur. As the coagulation cascade is activated, so are the complement cascades. These occur simultaneously. The platelets themselves release platelet derived growth factor,

transforming growth factor and insulin like growth factor. These act as early attractants for fibroblasts and other reparative cells (19, 27).

Inflammation

Once the coagulation processes are underway, the process that is common to all wounds- inflammation begins. Inflammation can be identified by the cardinal signs of redness, heat, swelling, pain and loss of function. The cells that are important in this process are the polymorphonuclear cells and macrophages. In this process, the clots, foreign bodies and bacteria are removed and all the substrates required for cellular matrix deposition are arranged. The wound becomes erythematous and edematous as futher humoral mediators are released. This then continues until the wound is covered by ectodermal or endodermal elements such as epithelium and blood vessels (19, 27). Macrophages further release basic fibroblast growth factor which is a chemotactic factor for fibroblasts and is additionally an angiogenic stimulant (20).

Matrix synthesis and deposition

Wound healing then progresses to a point where the wound is covered with epithelium and fibroblasts are laid down. These are the main cells that produce

collagen and the extracellular matrix. They also release glycosaminoglycans and elastin fibres. The presence of metalloproteinases in the matrix at this point is also a vital factor in the wound healing process. There is evidence to show that matrix derived metallo-proteinase 8 (neutrophil derived) is the predominant collagenase present in normal wounds that are healing (33). Collagen deposition reaches its peak at approximately 7 days after the initial wounding and production escalates to a plateau at 6 weeks. The crosslinking of the collagen matrix also occurs during this period and defects in this stage of the wound healing can cause apparently healed wounds to have abnormal characteristics, such as greater tissue elasticity and less strength (19, 27, 35).

Angiogenesis

This is defined as directed endothelial cell migration and growth and is stimulated by acidic and basic fibroblast growth factor that are produced by endothelial cells and macrophages. Any degradation of the basement membrane, which is a storehouse for the basic FGF causes release of this angiogenic factor (17). The blood vessels that are formed in response to these stimuli tend to thin walled, short-lived and fragile and can easily be disrupted.

This can contribute to poor wound vascularity in an otherwise granulating wound (19).

Epithelialization

This is directly stimulated by at least two growth factors epidermal derived growth factor (EGF) and keratinocyte growth factor (KGF) (20). EGF encourages cells to continue through the entire cell cycle and not stop differentiating. In the wound itself, it attracts epithelial cells, fibroblasts and endothelial cells. KGF, however, interacts with dermal fibroblasts and the extracellular matrix, contributing to epithelialization in full-thickness wounds by stimulating keratinocyte activity at the epidermal-dermal interface. Wound healing is also marked by laminin and collagen VII which are deposited as part of the extracellular matrix. However, the growth factors do not appear to themselves be affected by levels of laminin or even collagen type VII. The inference from this is that they do not have a feedback system controlling their expression (20, 34).

While this proceeds at the molecular level, wounds gradually remodel themselves till the fine layer of capillaries and vessels on the surface of the

wound regress and only the fibroblasts remain. Wound tensile strength also increases with time (19, 35).

Contraction

The destruction of soft tissue and the eventual repair always involve more than one type of cell being incorporated into the wound site and formation of a new connective tissue matrix. As skin overgrows the defect, the normal dermal appendages and fibrous structure serve to cover a significant area. However, interspersed along the edge of the healing area is scar tissue and this contracture differs from wound contraction. The greatest benefit of wound contraction is that the area required to be covered by scar contracture is minimized. However, the aesthetic and functional loss associated with wound contraction and scar contracture may be significant as can be seen in joint creases in burn wounds. As the fibroblast migration through the extracellular matrix occurs, the collagen fibrils are oriented into a streamlined shape. In conjunction with collagenase activity, there is normal orientation of collagen and extracellular matrix strengthening (19, 31, 35).

The myofibroblast is a connective tissue cell of mesenchymal origin with alpha-smooth muscle actin as its hallmark. Overwhelming evidence points to

the myofibroblast being the cell that is mainly responsible for wound contractions. Its presence helps in the re-orientation of the connective tissue around the wound edges. The dynamic force through the actin-myosin coupling effects is responsible for the wound and subsequent scar contracture (19). While being derivatives of the fibroblasts, myofibroblasts are also found to have a fibronexus which is a special entity that connects their cytoskeleton to the extracellular connective tissue matrix. This links the cell membrane between intracellular microfilaments and extracellular fibronectin.

Due to the presence of these specialised cells, the granulation tissue responds to drugs that stimulate smooth muscles and this could be a source for further discoveries into ways to promote rapid scar contraction. The presence of myofibroblasts at the wound site correlates with scar contracture. As the fibroblast activity decreases, myofibroblasts appear and promote scar formation. If there is a delay in apoptosis of the myofibroblasts, then their activity is prolonged, resulting in an abnormal balance extracelluar matrix, which results in a rigid, excessive scar (27, 34, 35). This, along with the presence of uncontrolled matrix deposition leads to hypertrophic scarring.

Remodeling

This is itself a vast topic and is the concern for a large volume of current research. At the heart of this is the collagen molecule, which is a triple helix. There are nine different types of collagen and abnormalities in their production can cause inherited diseases such as Ehler-Danlos syndrome and Marfan's syndrome. These syndromes are characterized by abnormalities in tissue tensile strength and healing.

Normal extracellular matrix consists of 80% type I collagen and 20 % type II collagen. In the initial part of remodeling, the levels of type III collagen rise for 3 to 4 days and then are followed by an increase in the levels of type I collagen. This is consistent with the early appearance and subsequent replacement of granulation tissue (rich in type III collagen) by collagen dominated ECM (rich in type I). Accumulation of collagen depends on the ratio of degradation versus synthesis and any healing wound will have lower degenerative enzyme levels compared to the synthetic enzymes.

This stage is extremely pertinent to wounds that convert from healing to non-healing ones. Any interruption in the synthesis of collagen or of its remodeling will disrupt wound healing. Some factors that have been implicated in this are

deficiency of ascorbic acid which is required in the production of prolyl hydroxylase (19, 20), a rate limiting enzyme in the manufacture of collagen.

Hypoxia has also been implicated in the disruption of collagen formation in a similar manner to ascorbate deficiency (19, 27). Hypoxia also accumulates lactate which stimulates collagen synthesis by collagen gene transcription and increasing the prolyl hydroxylase activity. However, trans-cutaneous oxygen tension is not uniform in the wound (19). The centre of the wound always has the lowest oxygen tension. In this particular area there is a decrease in the production of prolyl hydroxylase and therefore decreased collagen deposition. This explains why the centre of the wound is usually the last to heal (19, 21, 22). In the opposite way, fibroblasts which are exposed to higher oxygen levels, respond by increasing collagen synthesis.

Wounds that fail to heal

Like other abnormal wounds, chronic wounds have decreased levels of factors such as proteinases, cytokines and growth factors that would normally promote wound healing (2). In addition, there may be higher levels of TNF-alpha that decreases the activity of PDGF and EGF.

TNF-alpha is also important in that it stimulates the matrix metalloproteinases, a group of proteolytic enzymes, into higher levels of activity and therefore increases the wound breakdown rates. This leads to an imbalance in the rate of wound healing, with degradation of the collagen based matrix being broken down faster than it can be formed. This pushes a wound towards chronicity (2, 23).

The matrix metalloproteinases (33) further degrade the adhesive substrates and cytokine signaling molecules and reduce positive cell growth. The cellular degradation will also attract further inflammatory cells which pushes the wound towards further matrix degradation. Thus the entire process becomes one of negative cellular growth, and this process can be initiated at any time in the wound healing process.

There are some other external factors that can affect wound healing and which predispose to chronicity (31). These are discussed below.

Important factors affecting wound healing

Infection

This is probably the single most important reason for a wound to not heal (2). The presence of active infection as defined by a bacterial load of more than 100,000 colonies per cc will prevent flap closure and delay primary healing (27). Some bacteria which release thermolysins and express other factors will further contribute to the process of matrix degradation. In the process they may release factors such as pro-MMP-1 which attract matrix metalloproteinases which further speed up the degradation process.

Hypoxia

Hypoxia (22, 27) in the form of ischemia due to atherosclerosis, cardiac failure or venous ulcers can cause a decrease in the local tissue oxygenation. The presence of molecular oxygen in a bound from is essential for the production of collagen, especially the post-translational cross-linking and triple helix formation. Once the trans-cutaneous oxygen tension falls below 40 mmHg in the wound, the quality of the collagen that is formed is poor. It tends to be brittle and easily degraded. Further, any process that causes local vasoconstriction such as smoking will further hypoxia. While it is true that hypoxia itself stimulates angiogenesis, a fall below 40 mmHg will not be of any

benefit to the wound healing process. Anemia due to chronic disease can produce similar effects due to the reduced oxygen carrying capacity of the blood (27).

Diabetes mellitus

This chronic disease contributes to a large proportion of chronic wounds (1, 3,22, 28). It affects wound healing at all stages. Tissue hypoxia, repetitive trauma, neuropathic effects and accelerated atherosclerosis all play a role in the poor wound healing. Peculiar to diabetes mellitus is the fact that increased glucose levels also cause additional glycosylation of the collagen matrix, causing it to be more brittle and susceptible to degradation as compared to normal collagen.

Malnutrition

Malnutrition (23) is well known to delay wound healing, especially when the metabolism of the body is in a catabolic phase and protein degradation is more than usual, as in burns, chronic diseases and post-operative periods. Vitamins A

and K are also well known to be involved in wound healing, causing lysosomal membrane stability and preventing abnormal clotting through post-translational hydroxylative processes (27).

Minerals such as zinc and iron and a few micro-nutrients such as selenium and manganese also play a role in promoting wound healing at the micro-cellular level. These are important adjuncts and a normal diet will deliver adequate levels (27).

Advances in the treatment of chronic wounds

Since the disease burden is so high and the economic ramifications so vast, there is a lot of interest in chronic wounds and in any treatment that could potentially decrease wound healing time. The most promising ones are mentioned here.

Topical Applications

Since TGF-beta, PDGF and basic FGF are important cytokines in the process of wound healing, topical preparations of these were formulated in the mid-90's and marketed aggressively, purporting the rapid closure of wounds or various kinds. However, this has not been borne out by any major randomized controlled trial (40). Until the exact growth factor required for the particular, individual wound is known, the use of growth factors seems to be mis-directed (36).

Tissue engineering

This allows genetic manipulation of any injured tissue, including myocardium and liver as well as epithelium. These are materials composed of cultured dermal fibroblasts mated to a polyglactide scaffolding that provides

shape and support. Some of these also contain keratinocytes in the thought that they can grow into the host tissue. Once these are laid over the target area, they are replaced by the target tissues' own skin cells. Some of these are FDA approved and are in current use. The evidence for this is limited to a few randomized controlled trials (32).

Vacuum assisted closure devices

These devices have shown great promise in the rapid healing of chronic wounds. They isolate the wound in a vacuum and continuous or intermittent suction up to 120 mmHg is applied over the wound. The effects of this are to keep the wound dry, prevent secondary bacterial infection and promote growth factors by active mechanical recruitment. These are commercially available as suction devices. The disadvantage is that they keep the patient confined to the bed and prevent early ambulation. The initial trials have shown great promise (4).

Stem cell therapy

This new branch of medicine has held great promise and is the current area of much research (8, 12, 16). Stem cells can be bone marrow derived or embryonic in nature. Their pluripotent nature should naturally allow them to differentiate into various cells types, especially in areas of cellular loss.

However, unlocking the pathways of differentiation and culturing these cells towards active clinical use is still in an early stage.

Bone marrow derived stem cells

Stem cells can be embryonic or adult derived (8). They are known to incorporate themselves into tissue and to differentiate into required cell types. It is also well known that bone marrow in mammals contains progenitor or stem cells. Stem cells from bone marrow locally applied to chronic wounds have been shown to augment healing in animal studies (11, 24). A case report of 3 patients with chronic wounds that did not heal with routine and advanced treatment had complete closure of the wounds after local application of bone marrow (5, 26). There is, however no evidence in a randomized controlled manner that shows conclusively that local application of bone marrow derived stem cells augments chronic wound healing.

Stem cells derived from bone marrow can be hematopoietic or non-hematopoietic in origin. They differentiate into various cell lines depending on the inherent genetic make-up, but can convert to other cells based on various stimuli (16). These may include growth factors, electrical stimuli, chemo-attractants and various other cytokines, such as CD 34+. The exact stimulants required for differentiation into highly specialized cells such as keratinocytes still have to be accurately delineated (17).

Case reports and series where autologous bone marrow derived stem cells have been injected or applied onto chronic wounds have shown promising results although it has taken up to 3 years in some cases for complete wound resolution (5, 26). Animal models have also shown promising results (11, 24). Cells engrafted onto the backs of wounded nude mice have been shown to incorporate into the healing tissue by use of GFP labeling (24). This has also been done in human volunteers successfully.

There is also evidence to show that wounding is itself a stimulant for recruitment of these cells and that local cytokine release can serve as attractants for stem cells from the bone marrow as well as stem cells which are circulating peripherally (16, 17, 18). Therefore in theory the local stimulus should in fact cause these pluripotent cells to differentiate into the required fibroblasts, myofibroblasts and keratinocytes as well as dermal appendage precursors.

The absence of this occurring conclusively has led to another postulate, namely that the stem cells do not completely engraft, but instead fuse with the cells that are already present and prolong their life-span while increasing their size and function. This has been borne out in work done since 2004 (13, 14).

With this in mind, this thesis was devised to test in a randomized controlled manner whether the one time local application of bone marrow derived cells would improve the healing of chronic wounds over a fixed time period.

Methodology

Patients who were seen in the surgical outpatient department of the Christian Medical College, Vellore and those referred for management of chronic wounds to the Department of General Surgery, formed the study population. The duration of the study was between July 2006 and July 2008. This study received approval from the Institutional Review Board.

Sample size calculation:

Sample size equation for 2 independent groups was:

n\group_2(Z_-Z__)\2

where Z_ is the z-value for_ (Z value considered for p 0.05 is 1.96)

where Z__ is standard deviation in mean response

This came to a sample size of 63 cases and controls. However, given the rates of chronic wounds in the community (4.48\1000), it was calculated that a total number of 30 cases and 30 controls would suffice for a study power of 80%, in conjunction with the Department of Biostatistics, CMC, Vellore.

Statistical testing was done using the Student's independent two-tailed t- test for unequal sample sizes with equal variance. All statistical analysis was done SPSS 16.0™ and all graphs were created using Microsoft Excel Office 2007™ , with the assistance of the Department of Biostatistics, CMC, Vellore.

Inclusion Criteria:

- Male or female patients with chronic wounds that had not healed for 3 months or more.
- Wounds where primary closure could not be achieved.
- Wounds which were unsuitable for skin grafting.

Exclusion Criteria:

- Wounds with active infection defined as copious pus discharge from the wound.
- Wounds where distal pulses were not palpable
- Wounds more than 15 cm in any one dimension.

- Patients with Hansen’s disease.
- Patients who were pregnant.
- Patients who were on chemotherapy.
- Patients who were diagnosed with hematological disease.

After the patient was identified, he or she was consented for the study and recruited and given a unique identification number which was in series.

Their hemoglobin levels and blood sugar levels were also recorded .

The wound was measured and the longest and widest dimensions of the wound was measured to the nearest millimeter with a centimeter rule and documented. The measurements were from the edge of the wound.

The wound was also photographed using a Nikon S5 camera in autofocus mode and with a flash. The distance from the wound was kept as close to 1 meter as possible. The affected area was placed in anatomical position to allow for easy reproducibility in position for follow-up photographs (37).

Randomization

Block randomization was carried out in blocks of 10 using opaque standard envelopes and were opened in the presence of the investigator by a neutral third party.

Procedure

Cases:

Once the patient was randomized as a case, he or she had 10 cc of bone marrow aspirated from the posterior superior iliac spine under sedation and local anesthesia with strict aseptic precautions.

The bone marrow aspirate was centrifuged at 3600 rpm for 20 minutes and the buffy coat pipetted off.

Once the aspirate was prepared for injection, the wound bed was prepared by gently scraping it with the back of a forceps to encourage fresh surface bleeding (21).

The buffy coat was then loaded into a 5 cc syringe and using a 26 G needle, 2.5 cc was injected into the edges of the wound at four furthest points of the wounds and then at 4 intermediate equal positions between these equally.

The remaining 2.5 cc of the stem cell concentrate was sprayed onto the wound.

A saline dressing was done, adequate cotton pads applied over the wound and the patient was asked to follow up after 48 hours.

At 48 hours, the wound was opened, measured, photographed and a saline dressing done. Regular dressings were continued as required.

Controls:

The procedure for the control patients was exactly the same as for the cases in terms of consenting, randomization and follow-up. However, instead of undergoing a bone marrow aspiration and local application of the centrifuged cells, they received local injection of autologous peripheral blood (6). Once the wound bed was prepared, then 5 cc of peripheral blood was drawn from the brachial vein of the left forearm and injected into the wound in exactly the manner outlined for bone marrow concentrates in the case group.

Follow up

The patients were followed up at 1week, 2 weeks, 4 weeks (25, 39), 6 weeks and at 3 months following the first application of centrifuged bone marrow aspirate.

At each visit, the longest and widest dimensions (38) of the wound were measured from the edge and the wound photographed as for a surgical specimen.

The endpoints of the study were considered achieved when the wounds achieved complete closure, or became suitable for conventional treatment options such as skin grafting, primary closure or at 3 months when the wounds were no longer followed up due to time constraints (26, 32).

Data Analysis

Baseline demographics

Total number of patients recruited

- Cases 25
- Controls 24

Total number of patients who completed follow-up

- Cases 23
- Controls 24

Total number of patients lost to follow-up

- Cases 2
- Controls 0

Average age of patients:

- Cases 54 years and 3 months
- Controls 58 years and 7 months

Male to female ratio

- Cases 17:8
- Controls 15:9

Adequate glycemic control: All

Number of patients with bilateral wounds

- Cases 0
- Controls 2

Average wound age

- Cases 14.28 months
- Controls 10.21 months

Maximum wound age

- Cases 123 months
- Controls 37 months

Average initial wound size

- Cases 65.32 cm^2
- Controls 48.83 cm^2

Average percentage reduction wound size by 3 months

- Cases 36.40%
- Controls 27.32%

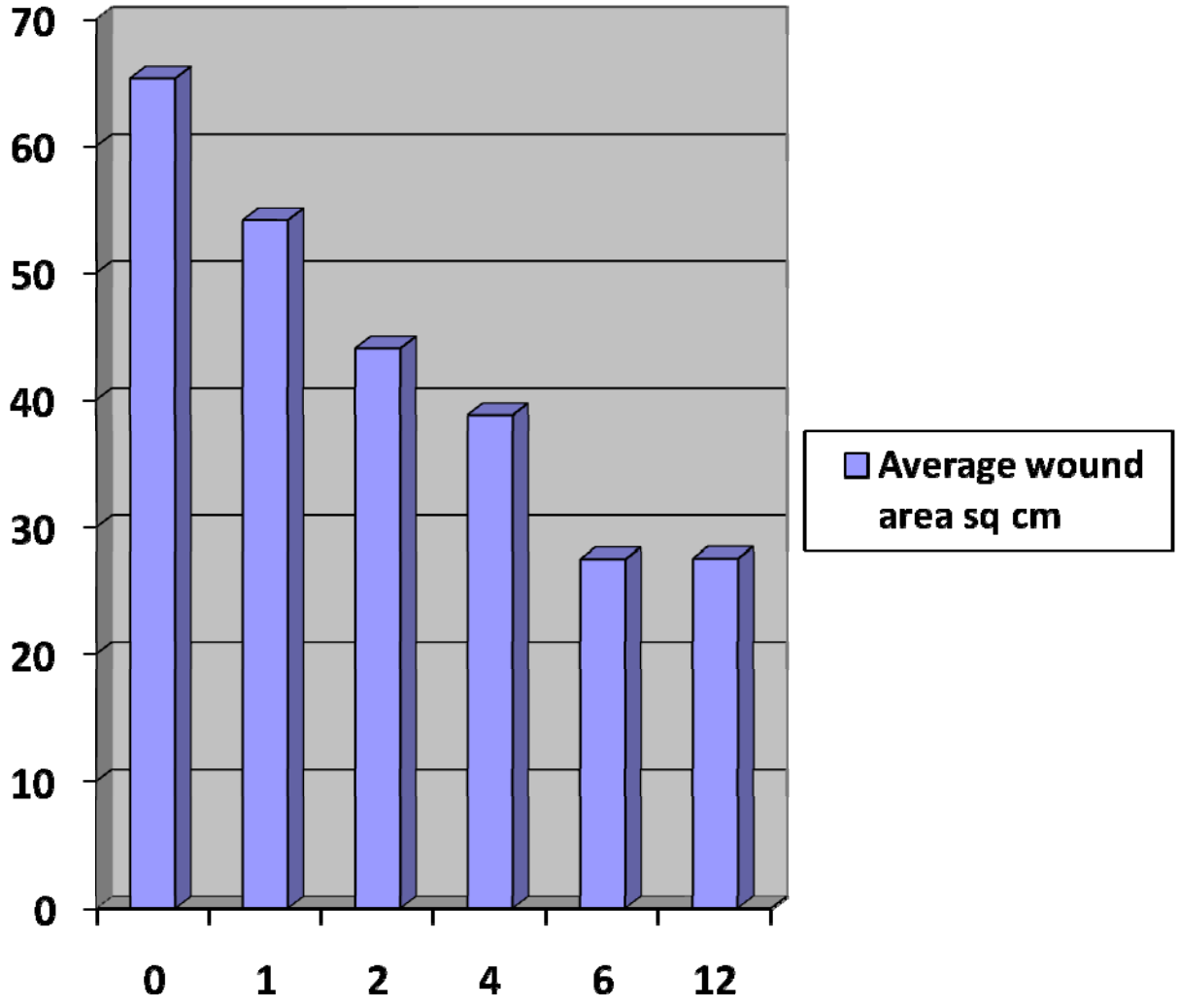
Number of wounds considered healed

- Cases 10
- Controls 7

Percentage decrease in wound size at 2 weeks

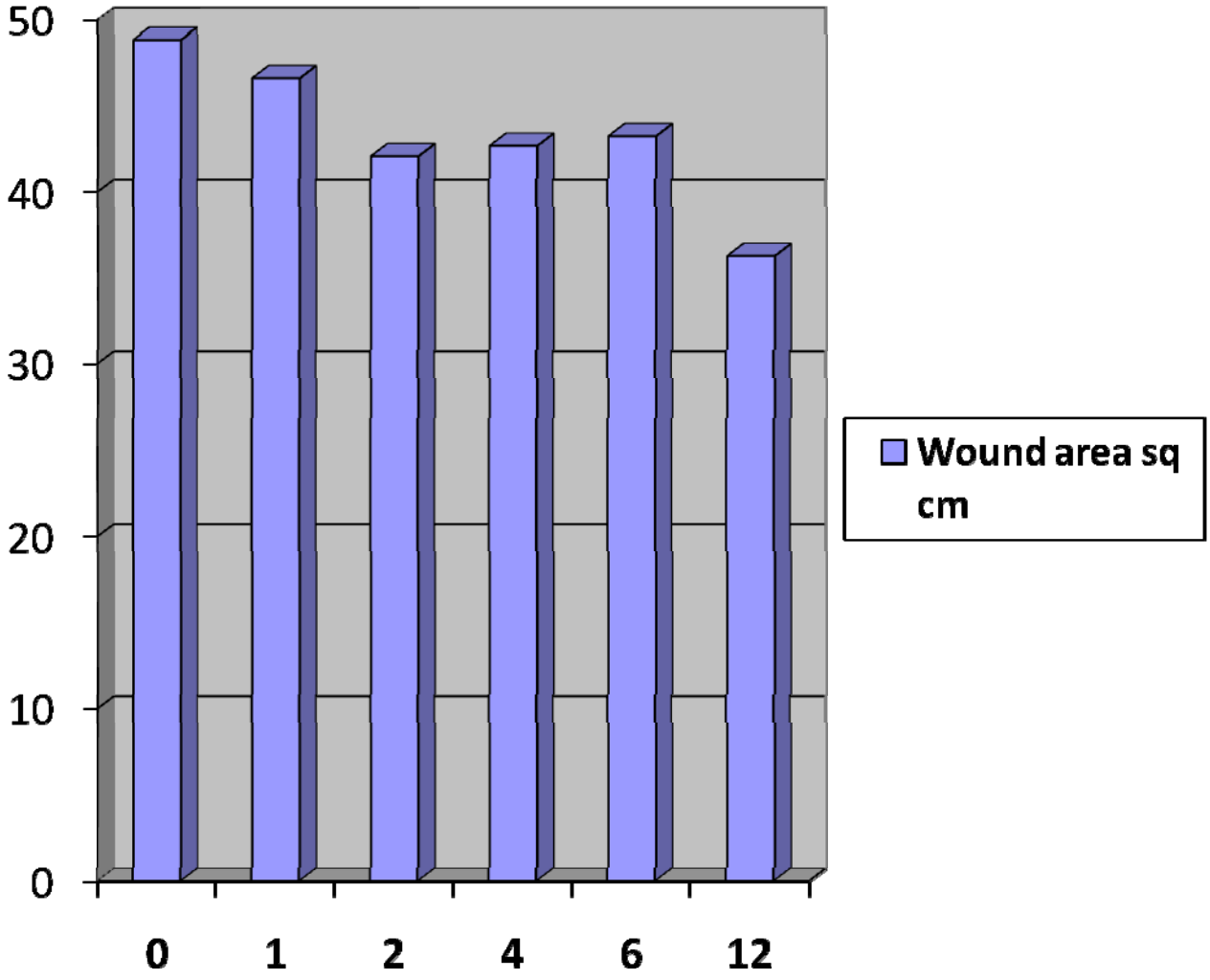
- Cases 17.0%
- Controls 4.84%

Cases



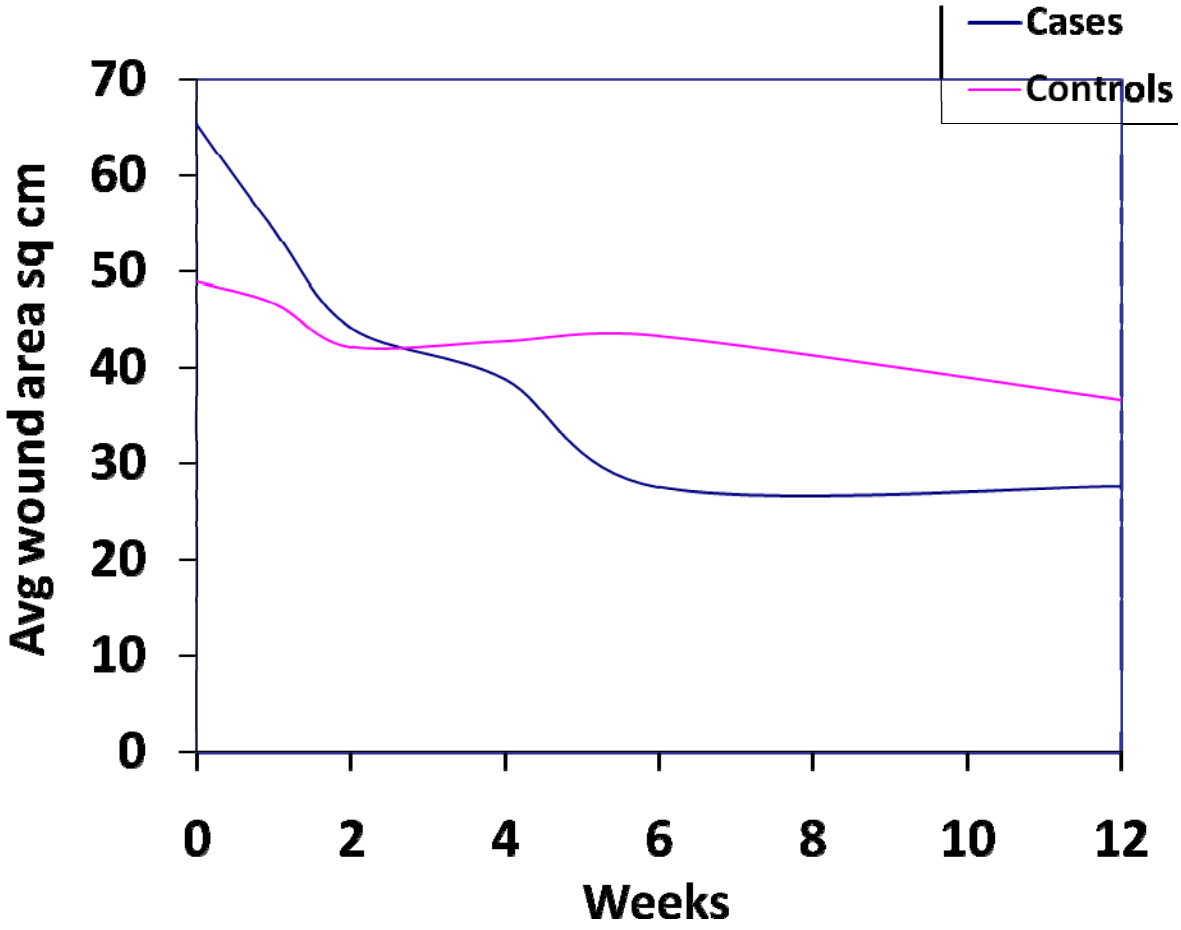
Graphical representation of changes in wound size over time in weeks following autologous bone marrow derived stem cell injection. (Fig 1)

Controls



Graphical representation of changes in average wound size with time in weeks.

(Fig 2)



Comparison of average wound size over time between cases and controls.

(Fig 3)

Typical case at first visit (Fig 4-1a).

Typical case at 6 weeks (Fig 4-1b).

Typical control at 0 weeks (Fig 4-2a)

Typical control at 6 weeks (Fig 4-2b)

New granulation tissue at injection sites (Fig 5)

Discussion:

The data presented shows clearly that there is a decrease in the wound area size in the control group in the first two weeks after the intervention in the form of the autologous bone marrow injection to the wound. The decrease in average wound size at 2 weeks in the cases is 17.4 % at 2 weeks as compared to 4.84 % in the control group. This is statistically significant ($p < 0.05$)

The overall decrease in the wound area at the end of the 3 months is 36.4% in the cases while in the control group it is 27.32%. This is not statistically significant ($p > 0.05$)

The wounds in the cases which were considered to be healed were 10 in number (40%), while the controls had 7 wounds which were considered to be healed (29.2 %). This is statistically significant ($p < 0.05$).

This study attempted to document whether the rate of wound healing in chronic wounds could be augmented by the local application of stem cells derived from autologous concentrated bone marrow aspirates.

1. It is clear from the data collected and analyzed that the local application of bone marrow derived stem cells to chronic wounds does indeed increase the rate of wound healing as measured by the decrease in

average wound size. This effect is maximal in the first two weeks after the injection and the effect appears to slowly decrease with time (Fig 1).

2. In the control group there is also an initial small decrease in the wound area indicating better healing, but this could perhaps be attributed to bias in an unblended study where the investigator takes greater interest in this wound knowing that it is part of a study. This is borne out by the rate of wound healing tapering off to a near plateau with time. Although the case group had a larger average wound size to begin with, there was no effect of this on the actual wound healing rate.

3. The mechanisms of the effects of the autologous bone marrow injection to the chronic wounds, while being effective in the first two weeks, are not clear. If the stem cells were indeed dividing and differentiating (8, 9, 12, 16) into the fibroblasts, keratinocytes and dermal appendages, the wound area curve should not have slowed. Furthermore the wounds should have epithelialized and closed of their own (26). In fact, none of the cases did this, and all the wounds that healed had undergone split thickness skin grafting once ready for it. This is borne out by work done on mice where the GFP labeled cells have been shown to have fused with the dermal cells in wounds on the backs of diabetic mice (11, 24).

4. The other possible mechanisms that may be augmenting healing over this short period of two to four weeks following the intervention are the local

cytokine factors (9,14,20) such as TGF-alpha, FGF, CD 34+ and CD 14 as well as a local increase in the oxygen tension of the wound (1,3,23). This could be the effect of the direct local application to the centre of the wound which is usually low in oxygen tension (1). This can be borne out by photographic evidence of new granulation tissue forming at the exact sites of injection in some of the wounds (Fig 5).

5. There were six control wounds which closed spontaneously, although this can be explained by the fact that the largest area in the sub-group was 8.32 cm^2 and with normal wound contraction rates they would have closed spontaneously in the absence of any confounding factor.

6. Since both case and control arms had adequate glycemic control (random blood sugar levels < 200 mg %) as evidenced by regular sugar monitoring either as inpatient or routine outpatient schedules, uncontrolled sugars cannot be said to be an impediment to healing. None of the patients had serum iron levels less than 7 mg % and anemia could therefore be ruled out as a confounding factor.

7. There is evidence to show that in diabetic wounds the rate of healing at 4 weeks is a robust indicator of whether the wound will become chronic or not (39). Figure 3 indicates that between 4 to 6 weeks is the time when the wound can be shown to have reached an obvious plateau in the healing rates, both in the cases and controls.

8. Since the effects of the one-time intervention in the form of the autologous bone marrow injection are maximal in the first 2 weeks and then taper off, it may be judicious to consider a second application at the end of this period in order to further augment wound healing. This will need further large-scale trials and further work needs to be done to clarify the exact mechanism of its action.

Final Conclusion

1. The local application of autologous bone marrow derived stem cells in the treatment of chronic wounds does indeed augment healing as evidenced by decreasing wound area following the intervention.

2. The effects are short-lived, the exact mechanisms unknown, but the positive effects on the wound are undeniable.

3. It is therefore possible to recommend this technique as an option in attempting to bring healing to otherwise refractory chronic wounds according to the inclusion criteria mentioned.

However, much work needs to be done to make this a more precise procedure.

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Appendix 1:

Patient information sheet.

Your doctor has informed you that you have a wound which has not healed for three months or more or that your wound is unlikely to heal in the usual way by itself. This is known as a chronic wound. He has also told you that there is a new method of treatment being evaluated which involves applying bone marrow from your body onto the wound in order to encourage the wound to heal more quickly. You are invited to take part in this study, but before you do so, it is important to understand why this research is being done and what would happen if you are involved. Please read the information below and do not hesitate to ask any questions.

What is a chronic wound ?

Any wound that does not heal within the usual duration of time is a chronic wound and for this study it is taken as any wound persisting beyond three months.

What is bone marrow and why is it useful?

Many bones in the body contain a substance called bone marrow which is the centre for blood cell manufacturing. It has cells called stem cells that can change into virtually any type of cell that the body needs. In this study we will be applying bone marrow onto and into the wound and assessing whether the wound heals more quickly than normal or not.

What is the purpose of the study?

In order to see whether applying the bone marrow actually helps the wound to heal faster or nor, we would like you to be part of a 'randomized' trial. Here you will be allocated to a group which either receives bone marrow to the wound or to a group where only blood is applied. Allocation is

done by chance, so you have an equal chance of being in either group. Neither the doctor nor you know which group you will go to until after you decide to take part.

Why are you chosen and do you have to take part?

You have been chosen because you have a wound that has not healed for more than three months. You do not have to take part. If you decide to do take part, you will be asked to sign a consent form. If you do not take part, you will continue will your usual treatment and the care you receive will not be affected in any way.

What will happen if I take part?

Once you sign the consent form you will be allocated to receive bone marrow or only blood. If you are allocated to receive bone marrow, your wound will be measured and photographed. Bone marrow will be taken from the back of your hip bone under local anaesthesia and injected and applied onto the wound. You will need to return for dressings as asked by your doctor for a total of five visits.

If you are allocated to the group that does not receive bone marrow, blood will be drawn from one of your peripheral veins and injected and applied over the wound. You will need to return for dressings as asked by your doctor for a total of five visits.

What are the side effects of taking part?

These are expected to be very minimal and rare since the blood or the bone marrow to be used is from your own body. However, the wound could get infected as could the site from where the blood or bone marrow is taken. The risk of this is very low.

Will my taking part be confidential?

Yes. If you take part in the study, the doctors involved will look at your charts and other documents. This study is coordinated by the Department of General Surgery, Christian Medical College, Vellore. The information about you is stored on a computer and in confidential charts. No one other than the personnel in the study will have access to your records.

What will happen to the results of the study?

The data will be analysed. If there is a significant result, then it will change the way in which chronic wounds are being treated.

Who has approved this study?

This study has been cleared by the Department of General Surgery and the Ethics committee, Christian Medical College, Vellore.

PATIENT CONSENT FORM.

Patient Hospital Number:……..

Name of researcher:……

1. **I confirm that I, the undersigned, have read and understood the infromation sheet dated July 2006 and have the chance to ask questions and clarify doubts.**
2. **I understand that taking part in this study is voluntary and that I am free to withdraw at anytime, without giving any reason and that doing so will not affect my medical and legal rights.**
3. **I understand that some of my medical notes will need to be looked at by responsible individuals from Christian Medical College, Vellore where the treatment is to be carried out. I give permission for these individuals to have access to my records.**
4. **I agree to take part in the study mentioned in the information leaflet.**

Name of Patient……Tel No……..

Address:……

……

Signature…… Date:……

Name of Witness:……

Signature…… Date:……..

Address of Witness:……

……

……..

Appendix 2

Data sheets:

Cases

Column1	Column2	Column3	Column4	Column5	Column6	Column7	Column8	Column9	Column10	Column11	Column12
Serial No	Hosp No	Wound age	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6		% reduction	
1	729350B	6	60.48	56	40.32	38.4				36.5	
2	978696C	4	8.6	8.4	7.98	7.4	6.8			20.93	
3	523187B	36	99.64	91.8	60.59	46.08				53.75	
4	542368C	8	52.46	49.2	48	45.82	42.12			19.71	
5	881761C	5	90.72	75.2	50.4	25.38				72.02	
6	789581C	7	120.4	113.6	99.56	74	57.12	49.6		58.8	
7	929636C	7	80	73.6	60.68	56.8	51.68			35.4	
8	094440D	4	14.08	12.9	12.6	10.64	9.99	8.88		42.05	
9	101135D	5	22.8	19.2	16.2	15.3	14.5	12.96		43.16	
10	`104485D	11	147.06	143.96	140.4	123.12	99.84			32.11	
11	113269D	8	40.32	36.6	31.8	30.16	26			44.75	
12	034716D	3	38.4	34.72	32.4	30	25.92			32.5	
13	131167D	4	99.84	93.84						6	
14		8	153.6								
15	595080C	6	55.4	52.3	50.2	46.2	38.72	32.7		40.97	
16	093790D	10	24.36	21.84	20.5	18.62	16.32	14.72		39.57	
17	727554C	123	146.88	138.72	135.34	122.88	116.56	110.4		24.84	
18	940820C	8	45.66	36.54	30.5	28.42	28.22	24.06		47.09	
19	157927D	18	36.54	34.76	30.2	28.88	26.13	25.12		31.25	
20	175146D	24	53.4	52.3	50.2	46.2	38.72	32.7		38.76	
21	919790C	7	86.28	73.6	60.48	58.8	51.68			40.1	
22	157311D	21	45.9	40.76	38.66	34.84	30.1	30		34.64	
23	418314B	5	21.22	18.26	13.29	13.01	12.44	10.76		49.29	
24	169080D	8	34.66	28.43	26.4	24.87	23.23	21.1		39.12	
25	850300C	11	54.24	48.54	46.21	44.2	40.06	39.76		26.7	
		14.28	65.32	54.2	44.11	38.8	27.47	27.52		36.40%	

Controls

Column1	Column2	Column3	Column4	Column5	Column6	Column7	Column8	Column9	Column10	Column11	Column12
Serial No	Hosp No	Wound age	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6		% reduction	
1	969731C	3	54.4	48.64	43.2	41.18	38.34	36.04		33.75	
2	960201C	2	22.26	20.8	19.38	17.28	15.64	14.85		33.28	
3	936829A	3.5	70.52	68	68.88	66.4	61.56	59.2		16.05	
4	822270C	5	4.6	3.96	3.2	1.5				67.39	
5	085786D	9	71.82	70.06	68.32	66	60.32	45.24		37.06	
6	131294D	1	8.32	8.32	3.12					67.18	
7	131282D	1	7.74	4.3	4.3					44.4	
8	097959D	7.5	118.8	112.64	99.2	92.72	88.8	86.14		27.49	
9	097959D	8	105.6	100.8	92.8	88	83.46	74.88		29.09	
10	957428C	9	9.6	9.28	8.96	8.37	7.54	6.72		30	
11	093790D	10	22.4	22	20.8	18.5	16.2	14.28		36.25	
12	579425C	12	72.36	65.52	62.22	58.58	52.64			27.25	
13	597425C	12	1.2	1.2	1.1					8.33	
14	165075D	16	23.65	22.21	20.1	20	18.65	17.24		27.1	
15	006204B	23	48.78	46.24	46.68	44.56	40.5	39.1		19.84	
16	529253C	12	44.24	42.21	43.67	42.27	40.58	40.26		8.97	
17	594004C	28	113.23	110.24	110.02	110.28	108.27	107.78		4.81	
18	029910D	2	3.6	3.2	2.87	2.08				42.22	
19	831366C	16	72.67	70.78	70.46	69.24	68.2	66.26		8.82	
20	831366C	18	76.88	74.24	70.2	68.46	68.26	67.29		12.4	
21	982080C	22	80.27	79.87	82.27	82.98	81.18	80.26		0.01	
22	153248D	3	32.27	32.02	31.35	28.46	28.33	27.1		16.02	
23	184299D	2	2.5	2.25	1.8	1.24				50.4	
24	126711C	37	104.28	100.48	98.96	97.26	95.29	96.54		7.42	
		10.21	48.83	46.64	42.14	42.74	43.3	36.63		27.32%	